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PERSISTENCE OF VIRUS AND BACTERIA IN SEAWATER

By William D. Won[1] and Harold Ross[2]

INTRODUCTION

Recent years have seen a rapid growth of interest in the preservation of ecological factors of our marine environment and a concurrent expansion of interest in the exploitation of coastal ocean areas for food production and sea bottom mineral resources. As a result of the former interests, large coastal communities now look to the ocean as an economical receiver of municipal sanitary wastes given only limited treatment, thereby forestalling or at least delaying the needs for costly advanced treatment of fresh water which might re-enter the consumer use cycle. It is apparent, however, that the ocean, particularly the Atlantic coastal shelf, comprised of relatively shallow water bordered by a megalopolis, cannot be considered an infinite sink for such wastes, and that the microbiological flora of these waters could be changed drastically by such dumping.

Examples of the need for study of the possible contamination of specific areas of ocean are readily apparent when considering the operations of offshore free divers during mineral exploration, repair work, or even recreational bathing at beaches quite remote from the "deep water disposal" point.

Aquanauts, in the recent Tektite program (7) resided and worked in an excrement-polluted marine environment for several periods. In the presence of such a background, incidents of acute skin and ear infections of undetermined etiology have been observed. (These unpublished incidents have been recorded in the Medical Log, Tektite II Program, Mission Number 3.2, May 11-18, 1970). Since

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the immediate possibility of more serious infections is a real concern to those working in such environments and the implication of the presence of viable infective organisms in spite of the massive dilution and adverse growth conditions present is also of concern to those proposing ocean outfalls for waste treatment plants, the writers investigated the survival characteristics of certain typical enteric organisms, (*Escherichia coli* and Echo 6 Virus) under a simulated marine environment.

Although seawater has been known to be bactericidal and virucidal, there are antagonistic factors capable of lowering these properties. The bactericidal property has been ascribed to several factors including: sunlight, salinity of the water (1); the presence of powerful oxidants (3); the presence of bacteriophage (6,9); and certain filterable, thermolabile agents of marine origin (2). Most of these factors, (salinity, chemicals in solution and heat labile, filterable toxic substances) were likewise virucidal (4). On the other hand, the addition of simple organic matter reduced substantially the bactericidal power of seawater (5,8), but reduction in activity against poliovirus was not apparent (4).

TABLE 1.—Summary Table of Materials Used

Test agent (1)	Test number (2)	Organic Supplements*: Each in Triplicate Flask						
		Feces (3)	Peptone (4)	Heart infusion broth (5)	Casamino acid (6)	Casitone (7)	Yeast extract (8)	Trypticase soy broth (9)
E. coli	1-7	X	X	X	X	X	X	X
Echo 6 virus	8-14	X	X	X	X	X	X	X

*Each test series paired with test agent in unsupplemented water as control.
Note: Test total number separate flask sets (3 each) = 14

This paper concerns studies on the protective effect of low concentrations of organic substances on the survival rate of bacterial and viral models, i.e., *E. coli* and type 6 Echo virus, representative of the enteric flora in sewage (see Table 1). Studies employing these models demonstrated a correlative relationship between the rate of bacterial survival and the degree of organic "pollution," and between the viral survival and the temperature of the water.

Material and Methods

Seawater.—Coastal ocean water samples were collected in sterile, clean plastic bottles at a 30-ft (9.2-m) depth, 0.25 mile (402 m) offshore from the Van Dam State Park about 150-miles (241-km) north of San Francisco, Calif. These samples were colorless and clear measuring 52° F (11° C) and pH 8.2. These were packed in ice for transportation to the laboratory, about a 3-hr trip. Sterilization was by pressure filtration through a Hercules ST-3 type pad.

Stock Organic Additives.—Bacto-peptone, Difco heart infusion broth, Bacto casamino acids, Bacto-casitone, Bacto yeast extract, and trypticase soy broth

(BBL) each at 5,000 ppm (5.0 mg/ml) were individually dissolved and autoclaved in seawater. The resultant loss of CO_2 here was not considered important since only small volumes of the stock solutions were used to supplement the final media. A 25% (w/v) fresh human feces was homogenized in seawater with a Waring Blender and autoclaved at 250° F (121° C) and 21 psig for 20 min.

Bacterial Preparations.—The *E. coli* (ATCC 9637) was grown in BBL trypticase soy broth for 24 hr at 85° F (30° C) yielding consistently 1×10^{10} organisms/ml The culture, which was to serve as an inoculum, was diluted with normal saline water to 10^6 organisms/ml; 2.0 ml were introduced into 200 ml of sterile seawater contained in a 500-ml erlenmyer flask, to yield a final concentration of 1×10^4 organisms/ml. Each flask was artificially polluted with an organic substance in concentrations ranging geometrically from 2.5 ppm to 500 ppm (2.5 μg/ml to 500 μg/ml). The suspensions were placed on a gyratory shaker operating at 200 strokes/min at 38° F-40° F (3° C-5° C) and 72° F (22° C). Samples were withdrawn at regular intervals and assayed for number of viable cells by dilution and plating on Bacto blood agar base plates which were then incubated at 85° F (30° C) for 24 hr.

For microscopic examination, drops of bacterial suspension were deposited on top of previous dried drops in order to increase the number of cells per field. The smears were stained with Gram stain in the conventional manner.

Viral Preparations.—A type 6 Echo virus, originally obtained from Fort Baker, San Francisco, was propagated in roller tube cultures of embryonic monkey kidney 104 (Microbiological Associates, Rockville, Md.) monolayers in Leibowitz's L-15 medium without serum. When cellular destruction was complete the cultures were frozen and thawed for 3 cycles to release the intracellular virus. The pooled virus material, which was to serve as the inoculum, was dispensed in 3 oz prescription bottles, 20 ml each, and stored at −94° F (−70° C). Virus titers were determined by the end-point dilution method by inoculating 0.1 ml of serial 10-fold dilutions in L-15 into each of four monkey kidney culture tubes per dilution. The cells were maintained in L-15 with 2% inactivated fetal calf serum and incubated in a roller drum at 95° F (35° C). The tubes were examined microscopically first at 72-hr intervals, and at 48-hr intervals thereafter for the rest of the 14-day incubation period. These preparations contained about $10^{9.4}$ 50% tissue culture infective doses ($TCID_{50}$) per 1.0 ml. For survival studies 2.0 ml of 10^{-2} saline dilution of the pooled virus preparation was added to each flask to yield about $10^{6.4}$ $TCID_{50}$/1.0 ml As in the *E. coli* suspensions each viral suspension received a concentration of an organic substance and aged on the gyatory shaker at 38° F-40° F (3° C-5° C) and 72° F (22° C) Samples were taken at regular intervals and assayed immediately.

Results and Analysis

Figs. 1–4 depict the protective influence of low levels of representative noncarbohydrate organic additives on a constant density of bacterial cells (1×10^4 organisms/ml) at 38° F-40° F (3° C-5° C). In all instances the apparent survivals were found to be greater in the organic additive-supplemented water than in the controls. However, as may be noted, the survival enhancing capacity of these substances differed. The substance most effective appeared to be Bactopeptone (Fig. 1) As is shown, bacteria in the control died off at a relatively

fast rate leaving approximately only 1.0% of the population surviving a 10-week exposure, after which time, viability was consistently undetectable upon assay. In contrast, better survival occurred when the water was supplemented with traces of peptone. Water supplemented with 125 ppm and 250 ppm (125 μg/ml and 250 μg/ml) of peptone elicited the highest survival and the longest persistence of the organisms (20 weeks). Similar results were achieved using heart infusion (Difco) supplemented water (Fig. 2). The survival response effected by other organics (fecal material, yeast extract, casitone, casamino acids, tryptic case soy, etc.) was less marked and all were of the same general magnitude (yeast and fecal material—Figs. 3 and 4 are typical). Increasing the concentration,

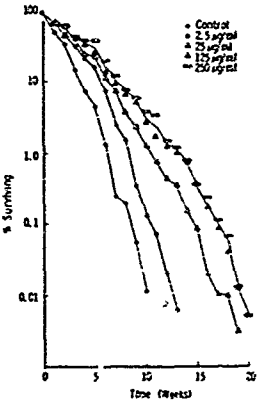

FIG. 1.—Survival Pattern of *E. coli* as Affected by Varying Concentrations of Peptone in Seawater at 35°-40° F (3° C-5° C)

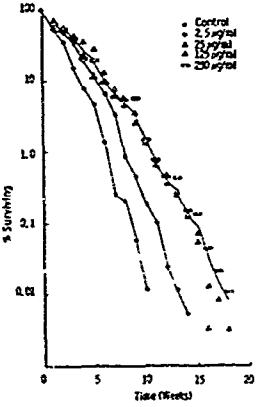

FIG. 2.—Survival Pattern of *E. coli* as Affected by Varying Concentrations of Heart Infusion Broth in Seawater at 38°-40° F (3° C-5° C)

by increments, to 1,000 ppm (1.0 μg/ml) did not significantly augment the protective effects of these materials.

Of additional interest was the observation that in the presence of organic substances, the morphology of the cells as well as the colonies appeared to be more stable. In seawater without additives the bacterial cells changed from the characteristic short rods to minute coccoidal forms in about 14 days. Cells suspended in water containing a low level of organic matter the coccoidal forms appeared at 22 days; whereas at the higher organic levels, these occurred at about 35 days

With respect to the colony development, a parallel, varying degree of effect was likewise noted. A small percentage of tiny colonies requiring an additional

24 hr of incubation to develop into countable sizes began to occur in the control populations at about the 3rd week and increased gradually up to maximum of about 35% at the 7th week. In contrast, these abnormal, tiny, slow growing colonies did not begin to appear in the high level peptone-supplemented water until after a 6-week exposure and did not reach maximum (35%) until the 13th week. It is possible that the coccoidal form is the consequence of an adaptive response to the stress imposed by an adverse environment. Further, a portion of the surviving population, more exacting in nutritional demand may have been selected.

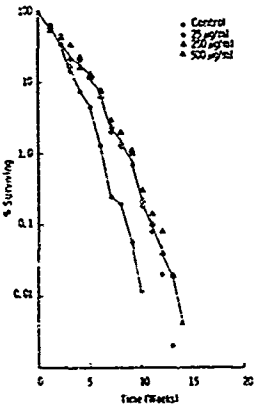

FIG. 3.—Survival Pattern of *E. coli* as Affected by Varying Concentrations of Yeast Extract in Seawater at 38°–40° F (3° C–5° C)

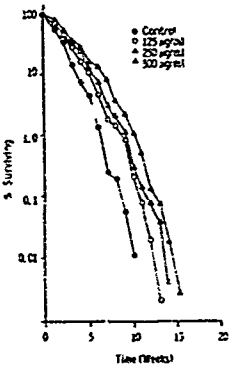

FIG. 4.—Survival Pattern of *E. coli* as Affected by Varying Concentrations of Fecal Material in Seawater at 38° F–40° F (3° C–5° C)

At 72° F (22° C), the effect of adding organic substance to seawater elicited a markedly different response, as shown in Fig. 5. Apparently, at this temperature the organic material completely overwhelmed the bactericidal properties of the seawater, permitting growth. For example, at 500 ppm (500 µg/ml), peptone, trypticase soy, and apparently feces, readily initiated and supported progressive bacterial growth for about 6 days, followed with a stationary phase leasting 3 weeks and then a sequential exponential decline proceeding at such a rate that viability in the order of about 50 organisms/ml persisted up to the 17th week. However, it has been established that, among the varied toxic substances, seawater contains a toxic principle which is thermolabile. Sterilization by autoclaving, filtration, or even aging affects its potency (1,2). Accordingly, in this respect, the protective activity of the organic additives observed may not be

indicative of a true state in nature; to some degree, it could likely be exaggerated.

Experiments with viruses showed that addition of various concentrations of peptone, casamino acids, yeast extract, trypticase, and fecal material to filtered

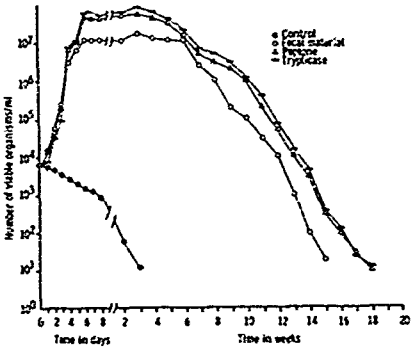

FIG. 5.—Influence of Organic Additives in 500 ppm (500 μg/ml) on Survival of *E. coli* in Seawater at 72° F (22° C)

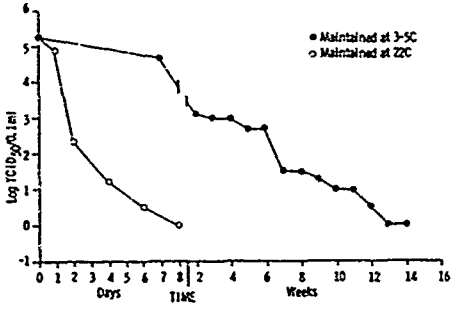

FIG. 6.—Inactivation Rate of Echo 6 Virus in Seawater as Function of Temperature

natural, aerated seawater did not appreciably change the survival capacity of the virus at either 38° F-40° F (3° C-5° C) or at 72° F (22° C). Fig. 6 compares the inactivation rate of the virus at 38° F-40° F and 72° F in unmodified, filtered

seawater. Inactivation was more rapid at 72° F than at 38° F–40° F. A cold environment appears to be more effective than organic pollutants in enhancing the persistency of the virus to provide a potential source of disease hazards for humans who come in contact with viral contaminated waters.

Summary and Conclusions

Survival of *Escherichia coli* and Echo 6 virus was studied in aerated seawater (shaking) under ambient conditions at 38° F–40° F (3° C-5° C) and 72° F (22° C). The addition of low concentrations of organic substances, including feces, enhanced bacterial survival at 38° F–40° F. At 72° F these organics became growth-promoting for *E. coli*, sustaining a 40-fold population increase, enabling viability to persist for 18 weeks. On the other hand, the addition of organic substances did not enhance viral survival. The initial inactivation rate for this virus was greater at 72° F than at 39° F–40° F.

Acknowledgment

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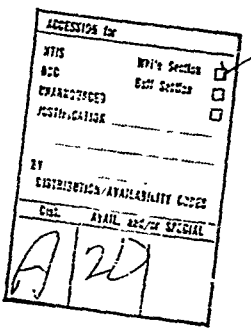

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KEY WORDS: Bacteria; Environmental engineering; Escherichia coli; Organic compounds; Sea water; Survival; Virus

ABSTRACT: At a temperature of 38° F - 40° F bacterial survivals (*Escherichia coli*) were significantly enhanced in seawater deliberately "polluted" with small concentrations of organic materials (25 ppm - 500 ppm) including peptone, heart infusion, casamino acid, yeast extract, trypticase soy, and human feces. In a warmer environmental temperature the survival enhancing property of these substances became growth promoting resulting in a 40-fold population increase followed with a marked increase in microbial persistence lasting at least 18 weeks. These substances were also capable of stabilizing cell and colony morphology. The same phenomenon was not observed in parallel experiments with Echo 6 virus. In this instance, cold temperature per se appeared more effective in enhancing survival time. Inactivation rate was gradual and the virus persisted for 14 weeks at 38° F - 40° F, contrasted sharply with an 8-day persistence at an environmental temperature of 72° F.

REFERENCE: Won, William D., and Ross, Harold, "Persistence of Virus and Bacteria in Seawater," *Journal of the Environmental Engineering Division*, ASCE, Vol. 99, No. EE3, Proc. Paper 9781, June, 1973, pp. 205-211